Aflatoxins M_1 and M_2 and Parasiticol: Thin Layer Chromatography and Physical and Chemical Properties

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A survey of known aflatoxin solvent systems for resolution of aflatoxins M_1 and M_2 on TLC plates revealed that the best system for determining aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2 is *iso*propyl alcohol-acetone-chloroform (5 + 10 + 85). Substitution of various alcohols for *iso*propyl alcohol in this system demonstrated that maximum resolution of M_1 and M_2 was achieved with *n*-amyl alcohol-acetone-chloroform (10 + 10 + 80); however, B_1, B_2, G_1, and G_2 migrated with the solvent front. When alcohol-chloroform (5 + 95) mixtures were investigated, *n*-propyl, *n*-butyl, and *tert.*-butyl alcohol + chloroform resolved M_1 and M_2 best but did not separate B_1, B_2, G_1, or G_2. Molar absorptivities of both M_1 and M_2 were determined in methanol, chloroform, acetonitrile, and acetonitrile-benzene (2 + 98). Relative fluorescent intensities of aflatoxins B_1, M_1, and M_2 were compared on both developed and undeveloped TLC plates. Fluorescent intensities of B_1 and M_1 on silica gel were nearly equal, and the intensity of M_2 was 1.4–1.5 times that of the other 2 aflatoxins. Water adducts of aflatoxin M_1 and parasiticol were prepared. The diacetate adducts of parasiticol were formed by treatment with acetic anhydride and concentrated HCl. Monoacetyl derivatives of M_1, M_2, and parasiticol were obtained by treatment with pyridine and acetic anhydride. Good resolution of the water-addition derivatives of B_1, G_1, parasiticol, and M_1 on TLC plates was achieved with *iso*propyl alcohol-acetone-chloroform (5 + 10 + 85).

Thin layer chromatography (TLC) has been an important technique for the determination of aflatoxins in extracts of agricultural commodities and products. Successful analyses depend on effective TLC solvent systems, accurate standards, and, finally, satisfactory confirmatory tests. Some procedures for aflatoxins other than M_1 and M_2 have been adopted as official by the AOAC (1, 26.001–26.061). No solvent system has been reported that separates M_1 and M_2 by TLC; therefore, analytical data given in the literature are based on the measurement of the combined M_1-M_2 fluorescence or aflatoxin "M." Previously, we studied solvent systems for improved resolution of aflatoxins B_1, B_2, G_1, and G_2 (2). Now we have investigated solvent systems for their ability to separate aflatoxins M_1 and M_2 on thin layer chromatoplates. Molar absorptivities used to determine concentrations of aflatoxins B_1, B_2, G_1, and G_2 in solutions have not been determined for M_1 and M_2 in solvents suitable for TLC standards. Official methods for confirming B_1 in sample extracts involve formation of water and acetate adducts (1, 26.054–26.055). Both water and acetate adducts (3) and the monoacetate derivatives (4, 5) of aflatoxin M_1 have been reported, but the latter 2 reports did not describe preparative methods in detail. Confirmatory tests for another toxic metabolite, parasiticol (6), also known as aflatoxin B_3 (7), produced by some strains of *Aspergillus parasiticus*, have not been attempted. Fluorescent intensities of M_1 and B_1 on TLC plates have been reported by Holzapfel *et al.* (5) and were used to determine concentrations of these aflatoxins.

In this paper, we report the separation of M_1 and M_2 on TLC plates, their molar absorptivities in methanol, chloroform, acetonitrile, and acetonitrile-benzene (AB) (2+98), and their fluorescent intensities on both developed and undeveloped TLC plates. We also describe the formation of water adducts of aflatoxin M_1 and of parasiticol, diacetates of parasiticol, and monoacetates of M_1, M_2, and parasiticol.

Experimental

TLC and Densitometry

TLC plates (20 × 20 cm) were coated with 0.5 mm silica gel (Adsorbosil-1, Applied Science Laboratories, Inc., State College, Pa.), air-dried 1 hr, and

activated 2 hr at 105°C. Five μl aflatoxin M_1-M_2 solution containing 2 μg M_1 and 1 μg M_2/ml AB was spotted at 4 locations across each plate along a baseline 1.5 cm from the bottom. Plates were developed by the ascending technique about 12 cm from the origin in unlined, unequilibrated, stainless steel tanks.

Initially, numerous solvent systems previously reported for TLC of aflatoxins were surveyed for their ability to resolve M_1 and M_2 on chromatoplates. After this survey, M_1-M_2 resolution was investigated by using various alcohols in an alcohol-acetone-chloroform (5+10+85) solvent system. Straight-chain alcohols from methyl to *n*-hexyl and *iso*propyl, *tert.*-butyl, and *iso*amyl alcohols were included. An identical study was made with alcohol-chloroform (5+95) systems.

Resolution of M_1 and M_2 was evaluated visually in a Chromato-Vue cabinet (Ultra-Violet Products, Inc., San Gabriel, Calif.) under 365 nm light during the original survey of solvent systems and densitometrically (2) (Photovolt Model 530 densitometer, Photovolt Corp., New York, N.Y.) for the alcohol systems. Resolution factors were calculated according to previously published procedures (2, 8). All resolution factors given are an average of the 4 values obtained from each plate.

Molar Absorptivity Studies

A stock solution of aflatoxin M_1 was prepared by dissolving 0.66 mg pure crystalline M_1 (9) in 3–4 ml acetonitrile, with intermittent heating in a water bath (50°C) and vigorous shaking before diluting to 10.0 ml with more acetonitrile. Absorptivities in this solvent were determined on a solution diluted to 6.6 μg/ml. A stock solution of M_2 in acetonitrile was prepared from 1.05 mg pure crystalline M_2 (9) by the same procedure and absorptivities were obtained on a solution diluted to 5.26 μg/ml.

Aliquots (0.5 ml each) of aflatoxin M_1 stock solution (66.0 μg/ml) were transferred to triplicate 5.0 ml flasks and evaporated to dryness under nitrogen. One M_1 dry film was dissolved in methanol, another dry film was dissolved in chloroform, and the remaining dry film was dissolved in 100 μl acetonitrile and diluted to volume with benzene to prepare 6.60 μg/ml solutions in methanol, chloroform, and AB for absorbance studies. Similarly, methanol, chloroform, and AB solutions (5.26 μg/ml) were made from M_2 stock solution (105.2 μg/ml). Ultraviolet absorption for all solutions was determined with a calibrated Beckman DB-G spectrophotometer, and extinction coefficients were calculated for wavelengths of maximum absorption according to the official AOAC method (1, 26.004–26.005, 26.009).

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Fluorescent Intensities of B_1, M_1, and M_2 on TLC Plates

Three μl aliquots each of standard aflatoxin B_1 and aflatoxin M_1 solutions (2.05 and 1.92 μg/ml AB, respectively) were spotted 9 times each across scored silica gel plates. Two types of silica gel (Adsorbosil-1; and silica gel G-HR, Brinkmann Instruments Inc., Westbury, N.Y.) were tested in conjunction with 3 solvent systems—water-acetone-chloroform (1.5+12+88) (2); methanol-chloroform (5+95); and *iso*propyl alcohol-acetone-chloroform (5+10+85) (10). Standard aflatoxin M_1 and M_2 solutions (1.92 and 1.50 μg/ml AB, respectively) were spotted by the same procedure on scored Adsorbosil-1 silica gel plates and developed in the following solvent mixtures—*iso*propyl alcohol-acetone-chloroform (5+10+85); *n*-propyl alcohol-chloroform (5+95); and *n*-amyl alcohol-acetone-chloroform (5+10+85). Fluorescent intensities of aflatoxins B_1, M_1, and M_2 on undeveloped plates were obtained by spotting scored Adsorbosil-1 silica gel plates with 3 μl aliquots of each standard in acetonitrile-chloroform (10+90). Each standard toxin solution was spotted 5 times on the same plate. This spotting solvent was used to attain zone diameters approximating those on developed plates.

All plates were scanned densitometrically on a Schoeffel SD 3000-3 spectrodensitometer. This unit includes dual-beam densitometer with 200 w xenon-mercury lamp, 200–700 nm quartz prism monochromator, variable-speed automatic-scanning TLC plate stage (0.25–8″/min), and 400–650 nm dual-wedge interference emission filters; density computer and 10″ strip chart recorder (Honeywell Electronik 194) with disk integrator for on-chart recording and automatic digital printout (Model 610). Maximum fluorescent values for B_1, M_1, and M_2 were recorded with the following densitometric settings: 362 nm excitation wavelength (1 mm band width); 3 mm slit widths incident to plate and interference filters; and 435 nm emission wavelength. The densitometer was operated in single-beam mode for all fluorescent readings.

Fluorescent intensities (integrator counts/ng toxin) for B_1, M_1, and M_2 were calculated from the recorded densitometric peaks, and intensity values relative to each other (M_1/B_1, M_2/B_1, and M_2/M_1) were determined.

Chemical Confirmatory Procedures for Aflatoxins M_1 and M_2 and Parasiticol

The official AOAC method (26.054–26.055) developed by Pohland *et al.* (11) was followed to make the water adduct derivatives of M_1 and parasiticol (0.1 μg each toxin) and the acetate adducts of parasiticol (0.1 μg). Monoacetyl derivatives of M_1, M_2, and parasiticol were prepared by treating 0.1 μg each

in foil-capped vials with 1 drop pyridine and 250 μl acetic anhydride. The solutions were mixed vigorously, heated 10 min on a steam bath, and evaporated to dryness under nitrogen. Each derivative (water adducts and acetates) was redissolved in 50 μl AB, 10 μl was spotted on Adsorbosil-1 TLC plates, and plates were developed in *iso*propyl alcohol-acetone-chloroform (5+10+85).

Results and Discusssion

Many solvent systems currently being used for aflatoxin analyses are capable of separating M_1 and M_2: water-acetone-ether (1 + 3 + 96) (12); water-acetone-chloroform (1.5 + 12 + 88) (2); water-ethanol-benzene (upper phase) (19 + 35 + 46) (26.016(b)); *iso*propyl alcohol-acetone-chloroform (5 + 10 + 85) (10); acetic acid-acetone-benzene (10 + 10 + 80) (13); ethyl acetate-acetone-benzene (10+10+80 or 30+10+60); hexane-acetone-chloroform (20 + 10 + 85) (14). Although a complete survey of reported aflatoxin solvent mixtures was not attempted, a sufficient number were successful and one can assume there are others which will resolve these 2 toxins. Almost all systems noted above contain 3 components of which acetone is one, but, as shown later, these requirements are not necessary for separation of M_1 and M_2 on TLC plates. However, every 3-component system investigated that contained acetone and that satisfactorily resolved aflatoxins B_1, B_2, G_1, and G_2 also resolved aflatoxins M_1 and M_2.

All efforts were unsuccessful to improve M_1-M_2 resolution with these systems by varying solvent ratios, by substituting benzene for chloroform or vice versa, or by combining solvent mixtures. Variation of solvent ratios caused changes in mobility of toxins on plates but not changes in separation. Generally, solvents containing benzene produced more compact zones than identical ones containing chloroform, but migration of B_1, B_2, G_1, and G_2 zones was less and G_2 and M_1 zones tended to overlap. Of the solvent systems listed, *iso*propyl alcohol-acetone-chloroform (5 + 10 + 85) (10) gave the best results (Fig. 1) and can be used for qualitative and, possibly, quantitative determinations of B_1, B_2, G_1, G_2, M_1, and M_2.

Other alcohols were substituted in the *iso*propyl alcohol-acetone-chloroform (5 + 10 + 85) system and the resolution factors were determined as follows: methyl, no resolution; ethyl, 0.41; *n*-propyl, 0.61; *iso*propyl, 0.57; *n*-butyl, 0.69; *tert.*-butyl, 0.53; *n*-amyl, 0.79; *iso*amyl, 0.73; and *n*-hexyl, 0.74. Separation of M_1-M_2 increased as the alcohol carbon chain lengthened until maximum resolution was achieved with the C_5 alcohol (*n*-amyl). In this solvent system, straight-chain alcohols separated the toxins better than their branch-chain isomers. An increased resolution factor (0.86) was attained with a solvent ratio of 10 + 10 + 80 for the best system, *n*-amyl alcohol-acetone-chloroform (Fig. 2). R_f values for M_1 and M_2 are approximately 0.5, and although B_1, B_2, G_1, and G_2 are not shown, they migrate with the solvent front.

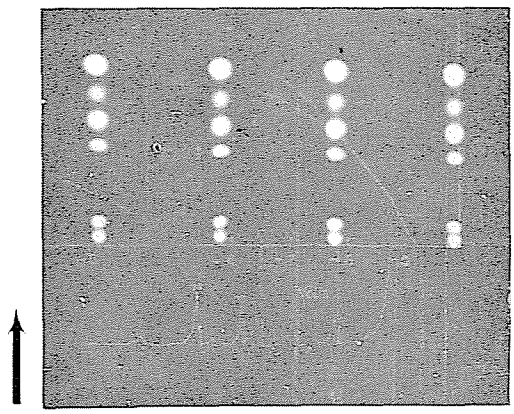

FIG. 1—Separation of aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2 (top to bottom) on Adsorbosil-1 silica gel TLC plate developed in *iso*propyl alcohol-acetone-chloroform (5+10+85).

Values of resolution factors were low when compared to those from a previous TLC study (0.90–1.0) with aflatoxins B_1, B_2, G_1, and G_2 (2). Visual evaluation of M_1-M_2 separations indicated that resolution was nearly complete and, therefore, the factors should be much higher. The method for determining resolution factors assumes that the fluorescent zones are perfectly symmetrical, but this assumption is evidently not correct. However, the method is still valid to determine which systems resolve the toxins best.

After substituting alcohols in the various systems indicated that this component was important for resolving M_1 and M_2, alcohol-chloroform (5 + 95) mixtures were investigated. Resolution factors for these systems were as follows: ethyl, no resolution; *n*-propyl, 0.80: *iso*propyl, 0.70; *n*-butyl, 0.80; *tert.*-butyl, 0.84; *n*-amyl, 0.71; *iso*-amyl, 0.52; *n*-hexyl, 0.48. M_1 and M_2 did not separate when either methyl or ethyl alcohol was

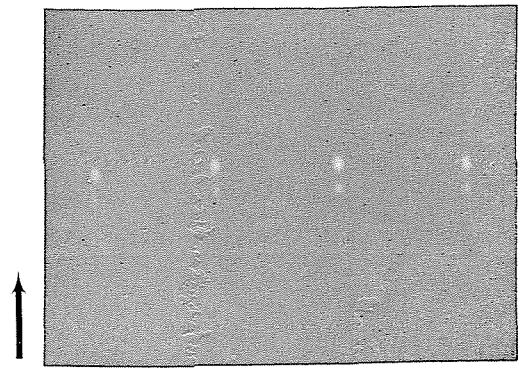

FIG. 2—Separation of aflatoxins M_1 and M_2 (top to bottom) on Adsorbosil-1 silica gel TLC plate developed in *n*-amyl alcohol-acetone-chloroform (10+10+80).

added, but resolution was achieved by addition of longer chain alcohols. Three alcohols gave about the same separation: *n*-propyl, *n*-butyl, and *tert.*-butyl. Resolution decreased sharply with amyl and hexyl alcohols. Plates developed in the best systems appear identical to that in Fig. 2.

Unfortunately, when plates were developed in alcohol-chloroform mixtures, a secondary solvent front was detected on most of the plates that restricted migration and separation of B_1, B_2, G_1, and G_2. These systems can be useful for samples, such as column fractions, that contain only M_1 and M_2. For quantitative determinations of samples containing all 6 aflatoxins, 2 solvent systems are usually necessary: *n*-amyl alcohol-acetone-chloroform (10 + 10 + 80) or *n*-propyl, *n*-butyl, or *tert.*-butyl alcohol-chloroform (5 + 95) for aflatoxins M_1 and M_2 and a system such as water-acetone-chloroform (1.5 + 12 + 88) (2) for aflatoxins B_1, B_2, G_1, and G_2. Since thin layer separations of aflatoxins are unpredictable, *iso*propyl alcohol-acetone-chloroform (5 + 10 + 85 or 3 + 10 + 87) may be satisfactory in some laboratories for quantitative determinations of all 6 aflatoxins.

Molar absorptivities of aflatoxins M_1 and M_2 in methanol, chloroform, acetonitrile, and AB are given in Table 1. The wavelengths of maximum absorbance and the extinction coefficients for these toxins in methanol are comparable to those reported in ethanol (5). A problem of solubility was encountered during the preparation of M_1 and M_2 solutions for molar absorptivity studies

Table 1. Molar absorptivity values for aflatoxins M_1 and M_2 in methanol, chloroform, acetonitrile, and acetonitrile-benzene (2+98)[a]

Solvent	Aflatoxin M_1		Aflatoxin M_2	
	λ_{max}	Extinct. Coeff.	λ_{max}	Extinct. Coeff.
Methanol	357	21,250	357	22,900
	265	14,150	264	12,100
	227	27,650	221	21,400
Chloroform	357	19,950	357	21,250
	267	12,950	264	11,650
	244	9,100	244	10,100
Acetonitrile	350	19,850	350	21,400
	265	13,750	264	12,050
	227	27,250	225	20,950
Acetonitrile-benzene (2+98)	345	17,450	345	18,750

[a] Calculated from maximum absorbance recorded by a calibrated spectrophotometer (Beckman DB-G) (26.004–26.005, 26.009). Each value represents an average of 2 determinations.

(and analytical standards). Neither crystalline M_1 nor crystalline M_2 is readily soluble in chloroform or methanol, but both can be dissolved in acetonitrile if heat and vigorous shaking are employed. Dry films dissolved more easily than crystalline products.

Relative fluorescent intensity values for B_1, M_1, and M_2 measured on both developed and undeveloped TLC plates are given in Table 2. In these studies, fluorescent intensities of aflatoxins B_1 and M_1 are nearly equal, which suggests an intensity ratio of 1:1 should be used to compare the 2 aflatoxins on TLC plates. In an earlier study, Holzapfel *et al.* (5) found a ratio of 3:1 (M_1:B_1), using silica gel G (Merck) and methanol-chloroform (3 + 97). Although this type of silica gel was not tried, no significant differences were apparent due to the silica gel or solvent system used. Since standard solutions of M_1 are available, authentic M_1 should be used as the reference.

A photograph of a TLC plate spotted with water adduct and/or acetate derivatives of B_1, G_1, parasiticol, M_1, and M_2 developed in *iso*propyl alcohol-acetone-chloroform (3 + 10 + 87) is shown in Fig. 3. The derivatives produced dominant, characteristic fluorescent zones that can be easily identified, even when background fluorescence which accompanies some of the commodity samples is present. Attempts to make the acetate adducts of aflatoxin M_1 by the official

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Table 2. Relative fluorescent intensities of aflatoxins B_1, M_1, and M_2 on developed and undeveloped silica gel TLC plates[a]

Solvent System	Relative Fluorescent Intensity[b]			
	Adsorbosil-1			Silica Gel G-HR
	M_1/B_1	M_2/M_1	M_2/B_1	M_1/B_1
Water-acetone-chloroform (1.5+12+88)	1.01	—	—	1.09
Methanol-chloroform (5+95)	1.04	—	—	1.14
*Iso*propyl alcohol-acetone-chloroform (5+10+85)	1.00	1.51	1.51	1.10
n-Propyl alcohol-chloroform (5+95)	—	1.41	—	
n-Amyl alcohol-acetone-chloroform (5+10+85)	—	1.47	—	
Not developed	1.03	1.41	1.46	

[a] Determined by TLC and densitometry.
[b] Determined by comparing the individual fluorescent intensities (integrator counts/ng toxin) relative to each other. Each value represents an average of 18 values from 2 plates, except those for not developed which are averages of 10 values from 2 plates.

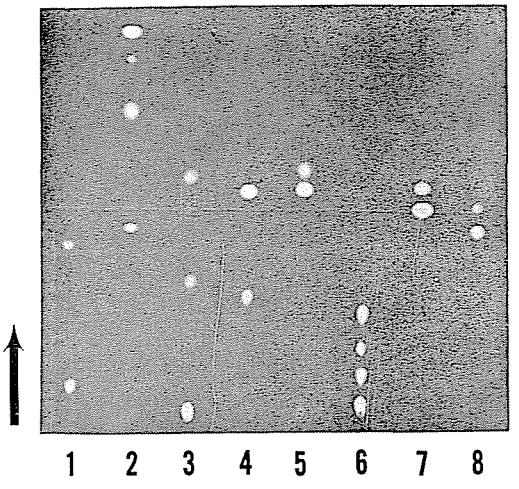

FIG. 3—Water and acetate derivatives of B_1, G_1, parasiticol, M_1, and M_2 spotted on Adsorbosil-1 silica gel TLC plate and developed in *iso*propyl alcohol-acetone-chloroform (3+10+87).

The derivatives are shown in their respective channels (top to bottom) as follows: channel 1, parasiticol and parasiticol water adduct; channel 2, parasiticol monoacetate and parasiticol acetate adducts; channel 3, M_1 monoacetate, M_1, and M_1 water adduct (M_{2a}); channel 4, M_2 monoacetate and M_2; channel 5, M_1 monoacetate and M_2 monoacetate; channel 6, water adducts of B_1 (B_{2a}), G_1 (G_{2a}), parasiticol, and M_1 (M_{2a}); channel 7, B_1 acetate adducts; and channel 8, G_1 acetate adducts.

AOAC method (26.054–26.055) were not reproducible and, at best, only faint zones were detected. Holzapfel *et al.* (5) prepared the monoacetate derivative of M_1 with pyridine and acetic anhydride under conditions that left aflatoxin B_1 unchanged; therefore, the hydroxyl group was acetylated and not the terminal double bond. The procedure given in the *Experimental* section produced the M_1 monoacetate (Fig. 3, channel 3) under conditions that left both B_1 and G_1 unchanged. This procedure also provided a method to confirm aflatoxin M_2 by its monoacetate (Fig. 3, channel 4). Likewise, a monacetate is formed from parasiticol (Fig. 3, channel 2) which gives 3 derivatives that can be used for confirming this compound.

Acetone-chloroform (1 + 9), the developing solvent recommended for identification of derivatives on TLC plates (26.054–26.055), does not separate the water adducts very well. The solvent system *iso*propyl alcohol-acetone-chloroform (3 + 10 + 87) not only resolves these compounds (Fig. 3, channel 5), but also separates the individual acetate derivatives. Because mobilities of the water adducts are increased with this system, identification is easier. Only the water-addition derivative of M_1 (M_{2a}) (Fig. 3, channel 3) remains near the origin. The solvent ratio 3 + 10 + 87 was used for the TLC reproduced in Fig. 3 so that all derivatives could be visualized on one plate. The need to confirm the presence of parasiticol in samples will be rare; therefore, the solvent ratio 5 + 10 + 85 is recommended to achieve the best separation of the water-addition derivatives.

When 50 μl AB was used to dissolve the derivative residues for TLC instead of the recommended 20 μl, the residues were more easily dissolved (especially M_{2a}). Also, with the larger volume of solution, an excess of sample was available for additional TLC in other solvent systems. Even at low concentrations, 10 μl produced prominent, fluorescent zones that were easily identified.

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